1232

Gradient Elution of Disaccharides on a Stearic Acid–Treated Charcoal Column

NANCY HOBAN and JONATHAN W. WHITE, Jr.
Eastern Regional Research Laboratory, Philadelphia 18, Pa.

▶ **During investigations on the minor sugars of honey, a technique for the isolation of disaccharides, not separable by paper chromatography, was needed. This paper describes the separation by gradient elution of four such pairs of sugars: turanose-sucrose, isomaltose-gentiobiose, maltulose-nigerose, and melibiose-lactose. Four other pairs of sugars (maltulose-sucrose, maltulose-maltose, sucrose-maltose, and turanose-isomaltose), although separable on paper, were also separated by this method.**

PAPER chromatography has been valuable for separating many carbohydrate mixtures. However, in some cases, occurrence of several sugars having similar R_f values makes isolation by this technique difficult or impossible. This method presents many experimental problems, such as uneven solvent fronts, sensitivities to temperature change, considerable losses of the original material, and, in the case of oligosaccharides, the time necessary for adequate separation. Impurities from the cellulose contaminate the sugars during the extraction process (*13*) and interfere with the determination of physical properties. Because of these limitations, many workers have modified the paper method or used other chromatographic procedures.

Bayly and Bourne shortened the separation time by converting the disaccharides into the *N*-benzylglycosylamine derivatives directly on paper. This accelerated the spot travel by decreasing its affinity to the cellulose-water phase (*5*). Tu and Ward obtained excellent separation of several disaccharides having similar R_f values by using a thermocolumn (*17*). Foster used ionophoresis successfully for separating such pairs as maltose and cellobiose (*9*).

Gradient elution, where the concentration of eluent is increased continuously, has been applied to various mixtures with success (*4, 8, 10, 14, 15, 20*). Improved separation over stepwise elution is achieved by reducing the tailing of zones (*3*). Alm (*2*) used gradient elution on a charcoal column treated with stearic acid for separation of carbohydrates, demonstrating that adjacent members of oligosaccharide series may be clearly separated. He did not attempt separations of sugars of the same molecular weight. This paper describes an application of the gradient elution method where, by continuously increasing the concentration of ethanol in a system, disaccharides having similar R_f values are separated. In some cases (Figure 2), separation is complete while in others slight overlapping of the zones occurs.

EXPERIMENTAL

The eight pairs of disaccharides studied are shown in Table I with their R_f values. All samples were commercial sugars, except isomaltose, which was obtained from the enzymic hydrolyzate of NRRL B-512 dextran; maltulose, which was prepared by the isomerization of maltose by lime water (*19*); and nigerose, which was obtained by the hydrolysis of nigeran (*21*).

Apparatus for Microgram Quantities. The apparatus (Figure 1) was similar to that described by Parr (*16*) and Bock and Ling (*6*). It consists of a mixer, which is equipped with a magnetic stirring bar, a reservoir, and a column. The mixer has twice the cross-sectional area of the reservoir, and the two vessels are connected by glass tubing. This connection is placed 30 mm. above the bottom to minimize backflow of solvent to the reservoir, which is caused by the continuous stirring of the magnetic bar in the mixer.

Table I. Pairs of Disaccharides Studied

Disaccharide	R_f Value[a]	Disaccharide	R_f Value[a]
Turanose	0.27	Maltulose	0.21
Sucrose	0.26	Sucrose	0.26
Isomaltose	0.14	Maltulose	0.21
Gentiobiose	0.14	Maltose	0.16
Maltulose	0.21	Sucrose	0.26
Nigerose	0.18	Maltose	0.16
Melibiose	0.14	Turanose	0.27
Lactose	0.15	Isomaltose	0.14

[a] Determined on Whatman No. 1 paper, at 23° C., with 1-propanol–ethyl acetate–water, 7:1:2.

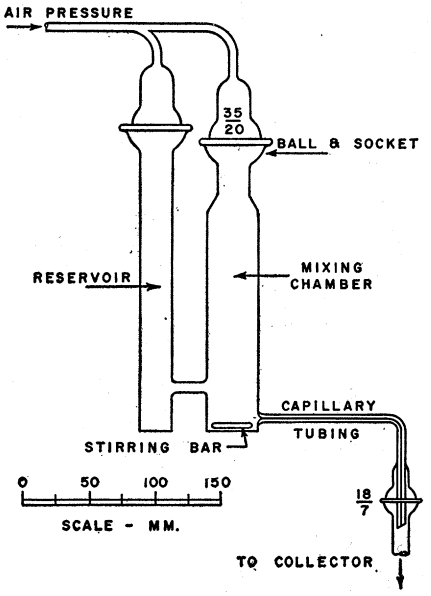

Figure 1. Apparatus for separating microgram quantities by gradient elution

Gradient. Successful production of a smooth gradient with this device depends upon the careful addition of ethyl alcohol to the reservoir. The water (145 ml.) was added to the system and then 50 ml. of 33% ethyl alcohol were layered carefully on it, using a separatory funnel with a reversed tip. This brought the meniscus between the alcohol and water layers in the reservoir slightly above the connection between the two vessels. During operation, a gradient from 0 to 6 to 7% ethyl alcohol was produced over a range of 140 fractions.

Preparation of Column. The adsorbent used was Darco G-60 charcoal (Atlas Powder Co., New York) treated with stearic acid as described by Alm (*2*). Each gram of charcoal adsorbed 200 mg. of stearic acid. A column, 9 × 220 mm. was packed with the charcoal mixture in a thick slurry and allowed to settle to 120 mm. under suction. After a 5-mm. layer of Celite 545 (Johns-Manville, New York, N. Y. was added to the top, it was washed with 500 ml. of water and stored in 50% ethyl alcohol. Prior to use, the alcohol was eluted with water and approximately 2 mg. of each known sugar of the pair were dissolved in water and applied to the column. It was then attached to the mixer, air pressure (5 p.s.i.) was applied to both reservoir and mixer, and 10-drop fractions were collected automatically. Because of the increasing alcohol content, the volumes of the fractions varied from 1.3 ml. at 0% ethyl alcohol to 0.7 ml. at 7% ethyl alcohol.

Analysis of Effluent. Sugar content of selected fractions was determined with a stabilized anthrone reagent (Carbanthrone, Rymark Laboratory, Terre Haute, Ind.). Color was developed by heat of dilution in test tubes. Accuracy was to about ±5%. By using displacement spectrophotometry as described by Hamilton (*11*), the color was read in the test tubes used in the fraction collector. No matching of tubes or transfer of sample was necessary. A Lumetron photoelectric colorimeter using an interference filter (600 mμ) was used. Alcohol content was determined refractometrically in five fractions of each run and the remaining were evaporated and spot checked by paper chromatography using propanol–ethyl acetate–water (7:1:2) as the solvent for a 2-day run (*1*). The sugars were located

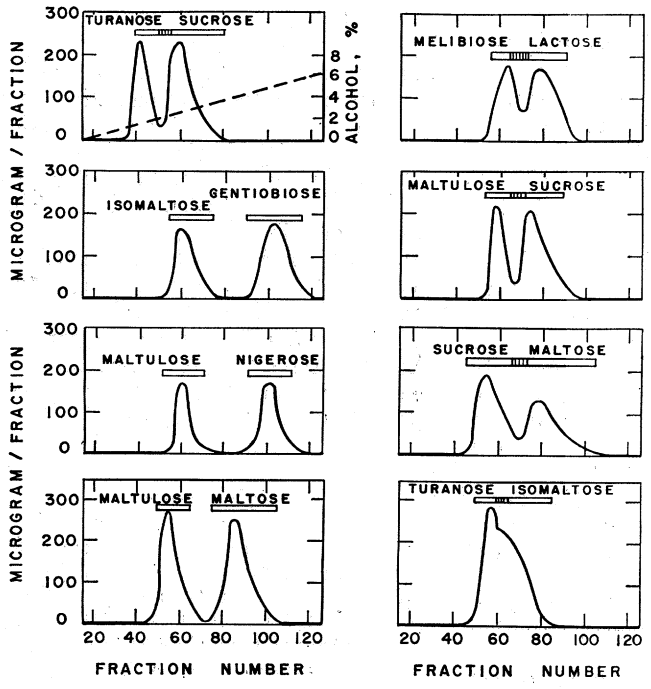

Figure 2. Separation of disaccharides by gradient elution in microgram quantities

---- Alcohol gradient of effluent, average of 8 gradients

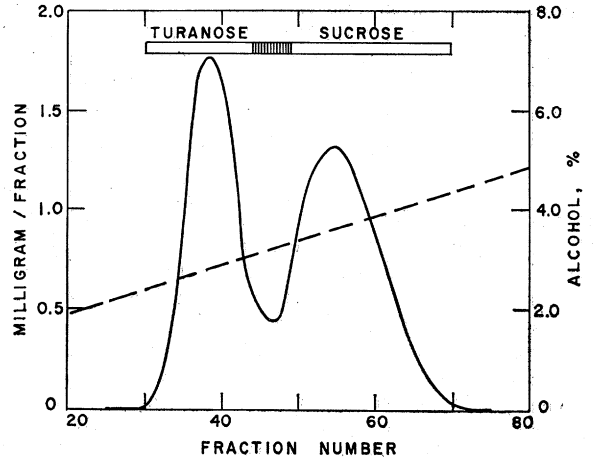

Figure 3. Separation of disaccharides by gradient elution in milligram quantities

on the papers by use of the specific color reagents, diphenylamine-aniline-phosphate (*12*) and triphenyltetrazolium chloride (*18*). Results are shown in Figure 2.

Apparatus for Milligram Quantities. For successful separation of these disaccharides in sufficient quantity for characterization, an apparatus 10 times larger was required. For this experiment, the constant-volume mixing system (*7*), was used. To minimize the convex nature of the effluent concentration curve produced by this mixer, only the first portion of the curve, which is essentially linear, was used. Because 1500 ml. of solvent were to be collected during the entire run, a round-bottomed flask, containing 5350 ml. of water, and a 2-liter filter flask were used for the mixer and the alcohol reservoir. The concentration of alcohol needed in the reservoir to produce a gradient similar to that of the small scale apparatus was calculated (*3*) to be 35%. A column, 20 × 290 mm., was packed to a depth of 260 mm. with the treated carbon mixture, and 20-mg. samples of both turanose and sucrose were applied to it. Fractions of 100 drops each were collected and their alcohol and sugar contents were determined by the methods for the small-scale apparatus. The results are shown in Figure 3.

RESULTS AND DISCUSSION

The method described permits the isolation of several disaccharides not separable by paper chromatography. Maltulose and nigerose, isomaltose and gentiobiose, and maltulose and maltose were completely separated (Figure 2). Although some overlapping of zones occurred with the remaining pairs, very little sugar was lost. In each experiment, no more than 10 fractions contained a mixture of the two sugars. For example, although the elution curve of turanose and isomaltose shows no minimum, the turanose, alone, was present in fractions 46 to 60, while pure isomaltose was found in fractions 65 to 85, with both sugars present in 61 to 64.

Individual sugars cannot be identified by their peak effluent volume (Figure 2). Differences in the alcohol concentration gradient between runs is largely responsible for this. However, the relative order of elution of the sugars is always the same.

The eight pairs of sugars separated by this method include four that could not be separated by paper chromatography—turanose and sucrose, isomaltose and gentiobiose, maltulose and nigerose, and lactose and melibiose; two that would require long periods of development for adequate separation on paper—maltulose and maltose, and maltutose and sucrose; and two that could be satisfactorily separated by the paper method—turanose and isomaltose, and sucrose and maltose. Even for those in the latter category, charcoal column elution avoids the experimental problems encountered in paper chromatography.

Comparison of the small and large scale separations of turanose and sucrose (Figure 2 and 3) shows favorable agreement. Although the effluent gradients differ, both separations of the sugars are adequate.

ACKNOWLEDGMENT

The authors wish to thank F. H. Stodola of the Northern Utilization Research and Development Division for supplying the sample of isomaltose and H. J. John of the Eastern Utilization Research and Development Division for constructing the apparatus.

PRINTED IN U. S. A.

LITERATURE CITED

(1) Albon, N., Gross, D., *Analyst* **77,** 410 (1952).
(2) Alm, R. S., *Acta Chem. Scand.* **6,** 1186 (1952).
(3) Alm, R. S., Williams, R. J. P., Tiselius, A., *Ibid.*, **6,** 826 (1952).
(4) Baker, C. A., Williams, R. J. P., *J. Chem. Soc.* **1956,** 2352.
(5) Bayly, R. J., Bourne, E. J., *Nature* **171,** 385 (1953).
(6) Bock, R. M., Ling, Nan-sing, ANAL. CHEM. **26,** 1543 (1954).
(7) Busch, H., Hurlbert, R. B., Potter, V. R., *J. Biol. Chem.* **196,** 717 (1952).
(8) Donaldson, K. O., Tulane, V. J., Marshall, L. M., ANAL. CHEM. **24,** 185 (1952).
(9) Foster, A. B., *J. Chem. Soc.* **1953,** 982.
(10) Grande, J. A., Beukenkamp, J., ANAL. CHEM. **28,** 1497 (1956).
(11) Hamilton, R. H., *Ibid.*, **25,** 399 (1953).
(12) Harris, G., MacWilliam, I. C., *Chem. & Ind. (London)* **1954,** 249.
(13) Huffman, G. W., Rebers, P. A., Spriestersbach, D. R., Smith, F., *Nature* **175,** 990 (1955).
(14) Lakshmanan, T. K., Lieberman, S., *Arch. Biochem. Biophys.* **53,** 258 (1954).
(15) Lederer, M., *Nature* **172,** 727 (1953).
(16) Parr, C. W., *Biochem. J.* **56,** xxvii (1954).
(17) Tu, Chen-Chuan, Ward, K. Jr., *J. Am. Chem. Soc.* **77,** 4938 (1955).
(18) White, J. W., Jr., *Arch. Biochem. Biophys.* **39,** 238 (1952).
(19) White, J. W., Jr., Eddy, C. R. Petty, J., Hoban, N., ANAL, CHEM. **30,** 506 (1958).
(20) Williams, R. J. P., *Analyst* **77,** 905 (1952).
(21) Yuill, J. L., *Chem. & Ind. (London)* **1952,** 755.

RECEIVED for review October 11, 1957. Accepted January 23, 1958. The Eastern Regional Research Laboratory is a laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. Mention of trade names in this paper does not imply endorsement of the U. S. Department of Agriculture over similar products not mentioned.